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Symbols, Abbreviations, and Acronyms

BPD	Bronchopulmonary dysplasia
CCSP	Clara cell secretory protein
COPD	Chronic obstructive pulmonary disease
CS	Cigarette smoke
FA	Filtered air (control)
H&E	Hematoxylin and eosin
IHC	Immunohistochemical
L_m	Mean linear intercept
MMP	Metalloproteinase
nAChR	Nicotinic acetylcholine receptor
PBS	Phosphate-buffered saline
qPCR	Quantitative polymerase chain reaction
RT	Room temperature
SP-B	Surfactant protein-B

ABSTRACT

Background: An epidemiological report suggested that cigarette smoke (CS) exposure during gestation increased the risk of bronchopulmonary dysplasia (BPD) - a developmental lung condition primarily seen in neonates and characterized by decreased alveolarization, angiogenesis and surfactant protein production, and may increase the risk of chronic obstructive pulmonary disease (COPD).

Objective: To investigate if gestational exposure to secondhand CS (SS) induced BPD and ascertain the role of nicotinic acetylcholine receptors (nAChRs) in this response.

Methods: BALB/c and C57BL/6 mice were exposed to filtered air (control) or SS throughout the gestation period or postnatally up to 10 wk. Lungs were examined at 7 days, 10 weeks, and 8 months after birth.

Results: Gestational, but not postnatal exposure to SS caused typical BPD-like condition: suppressed angiogenesis (decreased VEGF, VEGFR, and CD34/CD31), irreversible hypoalveolarization, and significantly decreased Clara cells, Clara cell secretory protein, and surfactant proteins B and C without affecting airway ciliated cells. Importantly, concomitant exposure to SS and the nAChR antagonist mecamylamine during gestation blocked the development of BPD.

Conclusions: Gestational exposure to SS irreversibly disrupts lung development leading to BPD-like condition with hypoalveolarization, decreased angiogenesis, and diminished lung secretory function. Nicotinic receptors are critical in the induction of gestational SS-induced BPD, and use of nAChR antagonists during pregnancy may block the CS-induced BPD.

INTRODUCTION

Bronchopulmonary dysplasia (BPD), first described in 1967 in premature infants with respiratory distress syndrome (Northway et al. 1967) is associated with fewer and enlarged alveoli, suppressed angiogenesis, lack or insufficient production of surfactant proteins (SP), and lung inflammation; however, impaired alveolar development and SP deficiency remains the main pathophysiological manifestation of BPD (Allen et al. 2003). BDP is the second leading cause of mortality among the infants born before 28 weeks of gestation (Callaghan et al. 2006).

Exposure to a wide range of chemicals and environmental toxicants during pre- and perinatal life may affect the maturation and function of the respiratory system (Pinkerton and Joad 2000). The risk of cigarette smoke (CS)-associated pulmonary complications is highest during fetal and early postnatal life (DiFranza et al. 2004), yet nearly one third of prospective mothers smoke during some stages of pregnancy (Hylkema and Blacquiere 2009). Epidemiological evidence and animal experiments suggest that parental, particularly maternal, smoking or nicotine exposure adversely affects the pulmonary health of the offspring, including higher risk for the development of allergic asthma, and airway remodeling and function (Rehan et al. 2009; Sekhon et al. 2001; Singh et al. 2009; Vrijheid et al. 2012). However, the question remains whether gestational and/or early postnatal exposure causes BPD. This is an important question because the infants with BPD are at increased risk of developing obstructive pulmonary diseases, such as COPD later in life (Narang 2010; Brostrom et al. 2010; Ali and Greenough 2012). In this communication, we show that gestational, but not early postnatal exposure of mice to secondhand cigarette smoke (SS) suppresses angiogenesis, alveolarization, and development of Clara and goblet cells without significant lung inflammation and the SS-induced effects on alveolar architecture are irreversible. Importantly, simultaneous exposure to the nicotinic

acetylcholine receptor (nAChR) antagonist mecamylamine (MM) blocks the effects of gestational SS on lung function and alveolar architecture.

METHODS

Animals

Pathogen-free BALB/c or C57BL/6 mice (FCR Facility,MD) were housed in shoebox-type plastic cages with hardwood chip bedding and conditioned to whole-body exposure in exposure chambers for 2 weeks before exposure to SS. The chamber temperature was maintained at 26 ± 2°C, and lights were set to a 12-hour on/off cycle. Food and water were provided ad libitum. All animal protocols were approved by the Institutional Animal Care and Use Committee. Experimental animals were treated humanely and with regard for alleviation of suffering.

Cigarette smoke generation and exposure

Briefly, 70-cm^3 puffs at the rate of 2/min were generated from 2R1 research cigarettes (Tobacco Health Research Institute, KY) by a smoking machine (Type 1300; AMESA Electronics, Switzerland). The smoke was captured from the lit end of the cigarette with a plastic manifold placed above it. Male and female mice were exposed to whole-body SS or filtered air (FA) for 6 hours/day, 7 days/week (total particulate matter 1.52 $\pm$ 0.41 mg/m^3) as described (Singh et al. 2011), approximately a dose of SS that a pregnant woman would receive by sitting in a smoking bar for 3 hr/day. Immediately after the birth of pups, smoke exposure was stopped; however, some gestationally FA-exposed pups were exposed to SS until 10 wk afterbirth (postnatal SS). See Supplemental Material for additional details.

Mecamylamine (MM) Treatment

Prior to SS or FA exposure, some breeder mice were subcutaneously implanted with ALZET miniosmotic pumps (pumping rate: 0.15 µl/hr) containing saline or MM (2.5 mg/mL), generating FA (control), MM+FA, SS, and MM+SS groups. Animals were sacrificed at indicated times.

Bronchoalveolar lavage fluid (BALF) collection

Established protocols were followed to obtain BALF from 10-wk old FA (control) and SS-exposed mice (Singh et al. 2009). The right lobe was lavaged twice with 0.8 ml sterile Ca^{2+}/Mg^{2+}-free PBS. The VEGF in BALF was determined by ELISA (Biosource-Invitrogen), according to the manufacturer's directions. Right lungs from some animals were frozen instantaneously in liquid N_2 and stored at -80 °C for Western blot analysis and total RNA for qPCR. See Supplemental Material for additional details.

Tissue preparation

After sacrifice, lungs were removed, inflated and formalin fixed at a constant hydrostatic pressure (Fritzell et al. 2009). Immediately after inflation, the trachea was ligated and the lungs were immersed in formalin for overnight. See Supplemental Material for additional details.

Hematoxylin and eosin (H&E) staining

Standard protocols were followed for H&E staining (Wang et al. 2001; Singh et al. 2011). The slides were examined with a Nikon Eclipse E600W microscope with a digital camera.

Morphometry

Mean linear intercept (L_m), was determined on H&E stained tissue sections using a Nanozoomer Digital Pathology (NDP) slide scanner (Hamamatsu K. K. Photonics). L_m was obtained by

dividing the NDP-generated total length of a line drawn across the lung section (scan resolution 20x) by the total number of intercepts encountered per lung section as described (Thurlbeck 1967; Knudsen et al. 2010). The slides were scored for crested alveolar septa using the NDP system with Visiopharm software (Hamamatsu K. K. Photonics, Japan) in five randomly computer-selected areas of each lung slide (16000 μm of alveolar area) of 7-Day, 10-Week, and 8-Months old mice.

Immunohistochemical (IHC) and Immunofluorescence staining:

Deparaffinized, and hydrated lung section (5 μm) followed by antigen retrieval were used for IHC and/or immunofluorescence (Tompkins et al. 2009) to visualize and quantitate SP-B$^+$ (1:1000 dilution; rabbit proSP-B antibody, Cat# AB3430; Chemicon), CCSP$^+$ (Clara cells marker, 1:50000 dilution; cat # 957, Vector), β-tubulin$^+$ (ciliated cells marker, rabbit anti-β-tubulin antibody from Abcam followed by Alexa 594-conjugated anti-rabbit antibody), SPDEF$^+$ (goblet cells marker, guinea pig anti-mouse SPDEF antibody, gift from Dr. Jeffrey Whitsett, Cincinnati Children's Hospital) and CD34$^+$ (anti-mouse CD34 antibody, 1:2000 dilution, Cat#:119301 from BioLegend followed by Alexa 555-conjugated anti-rat antibody) and/or CD31$^+$ (endothelial cells marker, rabbit anti-CD31, 1: 2000 dilution, Cat #: ab28364 from Abcam followed by DyLight 549-cojugated anti-rabbit antibody). Proximal and distal lung airways images were acquired using CCSP and SP-B stained lung sections (Nikon Eclipse E600W microscope) to access SS-effect throughout the lung (see Supplemental Material for additional details).

Trichrome Staining: Lung sections were stained with Masson's Trichrome (Sigma-Aldrich) to reveal collagen following standard protocol (see Supplemental Material for additional details).

Quantitation of IHC-stained SP-B$^+$, CCSP$^+$, and ciliated epithelial cells

The slides were scored for SP-B and CCSP-positive cells blind using the NDP system with Visiopharm software. Four randomly computer-selected areas of the each lung slide were quantified using 9000 μm of airways basal lamina. A similar approach was used for the quantitation of surface cilia stained with anti β-tubulin antibody.

Western blot analysis

CD34 were quantitated by Western blotting of the lung homogenates as described previously (Singh et al. 2011). The blots were probed with anti-CD34 antibody. Antibody-bound proteins on the blot were detected with enhanced chemiluminescence on an X-ray film. See Supplemental Material for additional details.

RNA isolation and quantitative PCR (qPCR)

Lung RNA was isolated as described previously (Singh et al. 2009). The lung expression of SP-B, SP-C, SP-A, CCSP, VEGFR2, and GAPDH were determined by qPCR using the One-Step RT-PCR MasterMix containing TaqMan probes and a specific-labeled primer/probe sets (Applied Biosystems).

Data presentation and statistical analysis

Data were analyzed using Graph Pad Prism software 5.03 (Graphpad Software Inc., San Diego, CA). One-way ANOVA was used to compare the mean between the groups using the Tukey post-hoc test that compares all groups at 95% confidence intervals. The student's t test was used for comparison between two groups. Results are expressed as the mean ± SD. A p value of ≤ 0.05 was considered statistically significant.

RESULTS

Prenatal SS exposure suppresses alveolarization

To determine the effect of prenatal SS exposure on BPD, lung sections from mice exposed gestationally to FA (control) or SS were examined microscopically at 7 days, 10 weeks, and 8 months after birth. H&E staining of lung sections suggested that gestational exposure to SS caused areas of hypoalveolarization in the lung and were seen in the early lung (7 days) as well as at 10 weeks and 8 months after the birth (Left panel, Figure 1A, 1B, and 1C, respectively). Changes in lung septation were quantitated by determining the L_m of alveoli. Compared to FA animals, the lungs from gestationally SS-exposed animals showed similar increases in L_m values (approximately 25%) at postnatal day 7, 10 weeks, and 8 months (Middle panel, Figures 1A, 1B, and 1C, respectively). Moreover, histopathology of the alveolar septae suggested that instead of elongated secondary septa seen in control lungs, SS-exposed lungs had an increased presence of crested secondary septae, indicating incomplete formation of secondary alveolar septa (Figure 1, arrows)—a feature also seen in human BPD (Ahlfeld and Conway 2012). In lung sections, these crested structures were counted and fold changes over FA-exposed animals plotted, showing significant increases in secondary crested structures in gestationally SS-exposed animals at 7 days, 10 weeks, and 8 months after birth (Right panel, Figures 1A, 1B, and 1C, respectively). On the other hand, postnatal exposure to SS (day 1 through 10 weeks) did not cause a significant change in alveolarization, and L_m values were comparable to control animals (Supplemental Material, Figure S1A). Thus, in utero but not early postnatal SS exposure impairs alveolar septation, leading to irreversible hypoalveolarization. To ascertain whether BALB/c mice were uniquely susceptible to gestational SS, we exposed C57BL/6 mice to gestational SS. Results from SS-exposed 7-day lung from C57BL/6 lung (Supplemental Material, Figure S1B) showed

morphometric changes similar to those observed in BALB/c mice. Thus both BALB/c and C57BL/6 mice develop impaired alveolarization in response to gestational SS.

In addition to impaired postnatal lung growth, BPD may cause variable interstitial fibrosis (Kinsella et al. 2006); however, collagen (trichrome staining) did not show any significant difference between FA- and SS-exposed 10-wk old lungs (Supplemental Material, Figure S2). Moreover, there was no indication of leukocytic infiltration in gestationally SS-exposed lungs (not shown). These results suggest that the SS-induced hypoalveolarization is not associated with significant lung fibrosis or inflammation.

Gestational SS exposure impairs the development of Clara and goblet cells but not ciliated epithelium cells

Downregulation of secretory proteins contributes to the pathology of several airway diseases, including BPD, and many premature infants are given a mixture of surfactant proteins to improve lung function (Kinsella et al. 2006; Merrill et al. 2011). To determine whether gestational SS broadly affected the airway secretory functions, we examined airway Clara and goblet cells in FA and SS-exposed mouse lung sections at 7 days post birth by staining for CCSP and SP-B (Clara cell markers) and SPDEF (goblet cell marker). As seen in Figure 2A (CCSP), Figure 2B (SP-B), and Figure 2C (SPDEF), the presence of Clara and goblet cells were significantly reduced SS-exposed lung. However, ciliated (β-tubulin-positive) cells (Figure 2D), not significantly changed by SS exposure in 7-day old lung. qPCR analysis also showed decreased mRNA expression of CCSP (Figure $2E_a$), SP-B (Figure $2E_b$), and SP-C (Figure $2E_c$). On the other hand, the mRNA expression of SP-A (Figure $2E_d$) and the number of β-tubulin-positive cells/μm basal lamina (Figure $2E_e$) did not show any significant changes between

gestationally FA- and SS-exposed lungs. Thus, prenatal exposure to SS impairs the development of Clara and goblet cells, contributing to the reduced production of surfactant proteins and airway mucus in gestationally SS-exposed lungs.

To determine whether gestational SS affected the airway secretory cells uniformly, the distribution of CCSP and SP-B in the airways was assessed by IHC staining in the proximal and distal airways. Results indicated that both proximal and distal airways were affected similarly by gestational SS (Supplemental Material, Figures S3 and S4).

Gestational SS impairs airway angiogenesis

Angiogenesis is a tightly regulated physiological process during embryogenesis (Breier 2000) and plays a vital role in the development of lung and airways (McCullagh et al. 2010), and new alveolar septa formation is closely associated with capillaries (Burri 2006). We evaluated lung vascularization by immunofluorescence staining for CD34, a transmembrane glycoprotein expressed primarily by endothelial cells and strongly present in alveolar wall capillaries (Pusztaszeri et al. 2006). Tissue sections were also stained for cell nuclei with DAPI. Compared to controls (FA), the SS-exposed lungs at 7 days after birth exhibited much weaker immunofluorescence (Figure 3A) for CD34, indicating a decreased number of endothelial cells in gestationally SS-exposed lungs. Western blots of the lung extracts from FA- and SS-exposed animals also showed that SS-exposed lungs had significantly reduced levels of CD34 immunoreactive protein (Figure 3B). Decreased angiogenesis in SS-exposed lungs was further confirmed by immunofluorescence for CD31 (Figure 3C), a major constituent of endothelial cell intercellular junction (Muller et al., 1989) and absent on non-vascular cells such as epithelium,

fibroblasts, and muscle cells (Newman et al. 1990). Thus, gestational SS exposure strongly suppresses/impairs vascular development in the lung.

Coordinated and timely release of angiogenic growth factors from respiratory epithelial cells promotes normal alveolar development (Thebaud et al. 2005). Although multiple factors affect angiogenesis, VEGF plays an important role in postnatal lung alveolar development as well as the maintenance of alveolar structures in the adult lung (Zhao et al. 2005; Carmeliet et al. 1996; Ng et al. 2001; Ruhrberg 2003; Kasahara and Tuder 2000). The expression of VEGF receptors increases during lung development, and most of the VEGF effects are mediated through VEGFR2 (Kalinichenko et al. 2001; Ng et al. 2001). We determined the expression of VEGFR2 by qPCR in the 7-day lung from FA- and SS-exposed mice. VEGFR2 expression was significantly reduced in the SS-exposed animals (Figure 3D). Moreover, the concentration of VEGF in BALF from SS-exposed mouse lungs at 10 weeks after birth was significantly lower than control lungs (Figure 3E). These results suggest that gestational exposure to SS causes angiogenic defects in the developing lung, and the decreased expression of VEGF and its main receptor VEGFR2 are likely to contribute to the defective angiogenesis of the lung in gestationally SS-exposed animals.

Mecamylamine blocks SS-induced effects on lung pathology

Nicotine is the major component of SS. Therefore, it was possible that the SS-induced BPD-like condition was regulated by nAChRs, and blocking these receptors would prevent the gestational SS-induced injury to the lung. We observed that while MM (nAChR antagonist) treatment during gestational period alone did not significantly affect alveolarization and L_m of 7-day old lung, it blocked the effects of gestational SS on alveolar septation (Figure 4A) and L_m values (Figure

4B). Furthermore, immunofluorescence staining for CD31 (Figure 4C) of the lung section, and qPCR analysis for VEGFR2 (Figure 4D) indicated that pretreatment with MM also normalized lung vascularization (CD31 expression) and the VEGFR2 in the 7-day old lung. Similarly, qPCR analysis indicated that MM restored the levels of SP-B (Figure 5A) and CCSP (Figure 5B), and also suppressed the SS-induced reduction in the airway SP-B staining (Figure 5C) and CCSP staining (Figure 5D). MM countered the effects of SS on $SP\text{-}B^+$ cells (Figure $5E_a$) and $CCSP^+$ cells (Figure $5E_b$). Moreover, MM restored CCSP- and SP-B-positive cells throughout the airways (Supplemental Material, Figures S3 and S4). Thus, MM treatment essentially blocks the inhibitory effects of gestational SS on alveolarization, vascularization, and secretory/surfactant protein production, indicating a critical role of nAChRs in the development of SS-induced BPD.

DISCUSSION

Increasing evidence suggests that in utero exposure to environmental toxins, including polycyclic aromatic hydrocarbons and CS/nicotine affect lung development and function (Perera et al. 2009; Rehan et al. 2009; Sekhon et al. 2001; Singh et al. 2011; Vrijheid et al. 2012). In addition, in rats maternal exposure to nicotine during gestation and lactation produced oxidative stress related lung impaired, including microscopic emphysema during adult life (Maritz and Rayise 2011). In humans, intrauterine exposure to CS has been deemed as a risk factor for BPD (Antonucci et al. 2004) but, to our knowledge, has remained unconfirmed. BPD is a disease whose etiology has not been fully established. Herein we present evidence that gestational, but not early postnatal exposure of BALB/c and C57/BL6 mice to SS causes BPD and the 7-day lung exhibit hypoalveolarization and significant increases in L_m ($\geq$23%) that is comparable to some patients with COPD/emphysema (Jacob et al. 2009).

Chronic exposure to CS is the most important cause of COPD/emphysema in humans, and the loss of alveolar surface is associated with increased concentrations of metalloproteinases (MMP) (Mocchegiani et al. 2011). MMP-12 is implicated in CS-induced emphysema (Belvisi and Bottomley 2003); however MMP-12 was not elevated in SS-exposed mice (not shown) suggesting that, whereas that classical emphysema is progressive and results from dissolution of preformed alveolar septae, the SS-induced BPD results from impaired formation of secondary alveolar septae and, in the absence of additional lung insults, is not progressive. This inference is further supported by the histopathology of SS-exposed 7-day old lung showing increased presence of crested secondary septa (i.e., incomplete formation of secondary alveolar septa). These results suggest that gestational SS-induced BPD results primarily from impaired alveolar maturation (Albertine et al. 2010).

Interaction between epithelial and vascular compartments is critical in alveologenesis and coordinated and timely release of angiogenic growth factors from respiratory epithelial cells promotes normal alveolar development (Thebaud et al. 2005). Fewer alveoli and microvessels are quintessential features of neonatal chronic lung disease (D'Angio and Maniscalco 2004) and pulmonary endothelium is closely apposed to the developing epithelium (Ng et al. 2001). VEGF is the most potent mediator of angiogenesis (Carmeliet et al. 1996; Ng et al. 2001) and plays a significant role in the pathophysiology of common respiratory disorders including acute lung injury, asthma, COPD, pulmonary fibrosis, and lung cancer (Papaioannou et al. 2006). The angiogenic effects of VEGF are primarily mediated through the VEGF receptors VEGFR2 (Kalinichenko et al. 2001; Ng et al. 2001). Consistent with the critical role of VEGF and VEGFR2 in BPD, the infants who die of BPD have little or no VEGF in their lung epithelium. BPD is also associated with decreased levels of angiogenic progenitor cells in cord blood (Baker

et al. 2012) and reduction of VEGF receptors in pulmonary vasculature (Thebaud et al. 2005). CD34 is present on endothelial cells, and is involved in leukocyte adhesion and endothelial cell migration during angiogenesis (Pusztaszeri et al., 2006). The expression of CD34 in the 7-day SS-exposed lung was severely reduced. Because CD34 is also present on progenitor hematopoietic cells (Civin et al. 1984), to ensure that SS affected the vascular endothelial cells, we also determined the expression of CD31 – a major constituent of endothelial cell intercellular junction (Muller et al. 1989). As with CD34, the lung expression of CD31 was much lower in gestationally SS-exposed animals than FA controls. Similarly, the lung expression of other parameters of angiogenesis, such as VEGF and VEGFR2 were significantly decreased in the 10-week SS-exposed lungs. Similarly, in preterm children with BPD, VEGF levels remain significantly lower than preterm children without BPD (Meller and Bhandari 2012). Decreased expression of VEGFR2 was also seen by microarray analysis of carotid arteries in monkeys exposed during gestation and early postnatal life to environmental tobacco smoke (Meller and Bhandari 2012). Together, these results suggest that decreased alveolarization in gestationally SS-exposed lung may reflect the reduction in angiogenesis that impacts lung vascularization.

Another characteristic of BPD is decreased production of CCSP and SP-B (Meller and Bhandari 2012). CCSP modulates immune responses, reduces lung injury in animal models, upregulates the expression of surfactant proteins and VEGF in the lung, and ameliorates BPD (Ramsay et al. 2001). SP-B lowers surface tension and prevents atelectasis and protects epithelial cells (Abdel-Latif and Osborn 2011), and its administration may improve lung functions in BPD (Logan and Moya 2009; Merrill et al. 2011). The expression of CCSP, SP-B, and SP-C is significantly downregulated in the gestationally SS-exposed 7-day lungs. However, the expression of SP-A surfactant protein was comparable between gestationally FA- and SS-exposed animals. This

disparity between the expression of SP-A and other surfactant proteins in SS-induced BPD is not clear; however, in the lung SP-A is also made by airway submucosal gland cells (Saitoh et al. 1998.)

There are three major epithelial cell types in the airways: ciliated cells, Clara cells, and goblet cells. Ciliated epithelium participates in the mucociliary clearance and expresses β-tubulin on the surface (Tompkins et al. 2009), while Clara cells and goblet cells express CCSP and SPDEF, respectively. IHC analysis clearly indicated that gestational SS did not significantly affect ciliated (β-tubulin^{+}) cells, but a drastic reduction was noted in CCSP^{+} and SPDEF^{+} cells in the airways.

Thus, the severe lack of secretory proteins in SS-exposed lungs may reflect impaired/delayed development of these cells in the SS-exposed lung. Indeed, the impaired airway secretory function such as mucus formation (Singh et al. 2011) and CCSP/SP-B was evident even at 10 weeks after the birth of gestationally SS-exposed animals (Supplemental Material FigureS5A/B). Decreased presence of CCSP-positive cells was observed both in proximal and distal areas of the airways, suggesting the effects of gestational SS on maturation and differentiation of type II epithelial cells. Additionally, CCSP stimulates VEGF and alveologenesis (Londhe et al. 2011; Abdel-Latif et al. 2011), its decreased expression of may contribute to impaired angiogenesis/alveologenesis in SS-exposed lungs.

SS contains many toxic chemicals including nicotine, and nAChRs are present on many non-neuronal cell types, including endothelial (Cooke and Ghebremariam 2008) and lung epithelial cells (Gundavarapu et al. 2012). Thus, it is possible that SS affects lung alveolarization, angiogenesis, and surfactant proteins through nAChRs. Moreover, in rats nicotine treatment

during gestation was shown to affect some aspects of lung development (Maritz and Rayise 2011). Treatment with the nAChR antagonist MM during the gestational period ameliorated the effects of gestational SS on alveolarization, angiogenesis, and airway secretory function. These results suggest that nAChRs are critical in SS-induced BPD. A surprising observation was that the in utero SS effects on some lung parameters were opposite of those observed in adult mice after CS/nicotine exposure. For example, exposure of adult mice to CS/nicotine suppresses allergic responses (Mishra et al. 2008), but prenatal exposure to SS strongly exacerbates allergen-induced atopy and Th2 polarization (Singh et al. 2011). Similarly, unlike its anti-angiogenic effects during gestational period, nicotine stimulates neovascularization (Cooke and Ghebremariam 2008) and airways mucus formation (Singh et al. 2011; Gundavarapu et al. 2012). A potential explanation is that long-term exposure to low levels of CS/nicotine may promote desensitization or loss of nAChRs, and these receptors may be important in regulating lung growth. Although currently we have no direct evidence to support this possibility, recent papers suggest that while short-term exposure to nicotine may promote angiogenesis and VEGF production, long-term exposures to nicotine may impair cholinergic angiogenesis and impair capillary sprouting (Konishi et al. 2010).

Although maternal smoking has been considered a risk factor for BPD in children (Antonucci et al. 2004), mice might be particularly sensitive to pro-BPD effects of SS. Alternatively, unlike humans, experimental mice are inbred and the presence of susceptibility factors on both alleles might make them more susceptible to CS-induced BPD. Nonetheless, this excessive susceptibility might make an excellent model to study the mechanism of BPD. Interestingly, postnatal SS had very little effect on the development of BPD. In view of recent human data suggesting that exposure to CS during first trimester is sufficient to increase the risk of asthma in

children (Vrijheid et al. 2012), CS-induced changes during early embryogenesis might be critical for BPD. Overall, the current study shows that exposure to environmental tobacco smoke during gestation interferes with alveolarization and promotes a BPD-like syndrome. These effects of SS are mediated through nAChRs and antagonists of nAChRs may have therapeutic value in blocking the effects of CS/nicotine on fetal lung development.

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Figure Legends

Figure 1. Gestational exposure to SS affects normal alveolar development. Representative sections (Left panel, 20X magnification) of lungs from 7-day old mice (**A**), 10-week old mice (**B**), and 8-month old mice (**C**). → shows blunted alveolar septal formation. Middle panels: L_m (estimates alveolar size). Right panels: fold crested alveolar septa. FA = filtered air; SS = secondhand cigarette smoke; data are means ± SD, $p \leq 0.05$ statistically significant (n = 5).

Figure 2. Gestational exposure to SS impairs the development of Clara and goblet cells but not ciliated cells. Lung sections (40x magnification) of 7-day old mice stained with anti-CCSP antibody (**A**), anti-SP-B antibody (**B**), anti-SPDEF antibody (**C**), Immunofluorescence image stained with anti-β-tubulin antibody for ciliated cells (**D**), qPCR analysis for CCSP ($\mathbf{E_a}$), SP-B ($\mathbf{E_b}$), SP-C ($\mathbf{E_c}$), SP-A ($\mathbf{E_d}$) and surface cilia/µm basal lamia ($\mathbf{E_e}$). FA = filtered air (control); SS = secondhand cigarette smoke; → shows stained cells in the airways; data are means ± SD, NS = not significant; * $p \leq 0.05$, ** $p \leq 0.0$, (n = 3-5).

Figure 3. Gestational exposure to SS inhibits angiogenesis. Representative lung (7-day old mice) immunofluorescence image (400x magnification) stained with anti-CD34/anti-rat conjugated Alexa 555. Sections are presented as nuclei (blue fluorescent), CD34 (red fluorescent), and merged image (blue and red fluorescent) for both FA and SS (**A**), Western blot: 70 µg lung homogenates (**B**); Representative lung immunofluorescence image stained with anti-CD31 (PECAM-1) (**C**), VEGFR2 by qPCR of 7-day old lung (**D**), and VEGF level in BALF (10-week old lung) by ELISA (**E**). FA = filtered air (control); SS = secondhand cigarette smoke; data are means ± SD, * $p \leq 0.05$, ** $p \leq 0.01$, (n = 5).

Figure 4. Gestational SS-induced effects on alveolarization and angeiogenesis are blocked by MM. Representative (H&E) 7-day old lung sections (40x magnification) (**A**), % L_m **(B)**, Representative immunofluorescence image of lung stained with anti-CD31 (PECAM-1)/anti-rabbit DyLight 549 (**C**), and qPCR analysis of VEGFR2 (**D**). FA = filtered air (control); SS = gestational secondhand cigarette smoke, MM+FA = MM alone without gestational SS exposure, and MM+SS = MM-treated mice exposed to gestational SS; data are means ± SD, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, (n = 5).

Figure 5. Gestational SS-induced effect on Clara cells is blocked by MM. qPCR of 7-day old lung for SP-B (**A**) and CCSP (**B**). Representative lung IHC image (40x magnification) stained for SP-B (**C**) and CCSP (**D**). $SP\text{-}B^+$ cells/μm basal lamina ($\mathbf{E_a}$), and $CCSP^+$ cells/μm basal lamina ($\mathbf{E_b}$). FA = filtered air (control); SS = gestational SS; MM+FA = MM alone without gestational SS exposure; and MM+SS = MM-treated exposed to gestational SS; → directed toward positive cells in the lung airways; data are means ± SD, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, (n = 5).

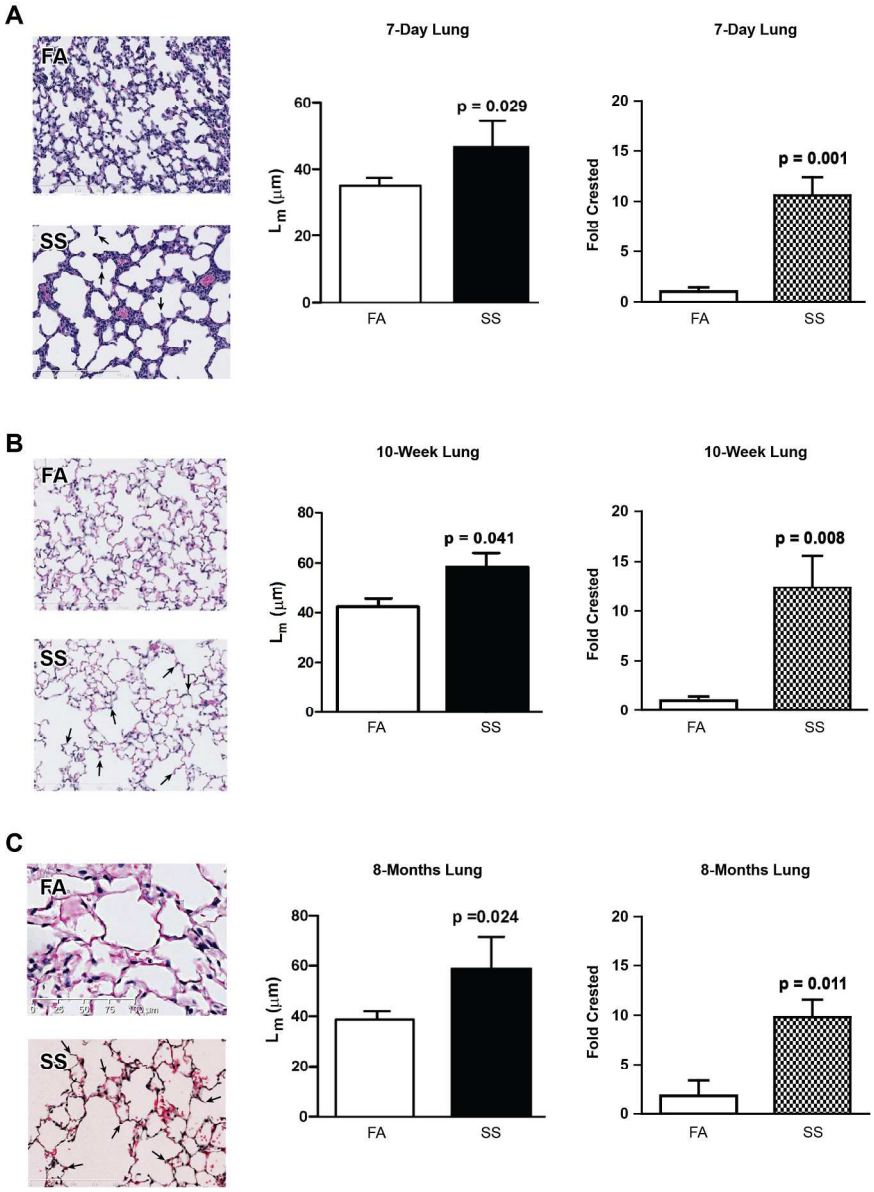

Figure 1

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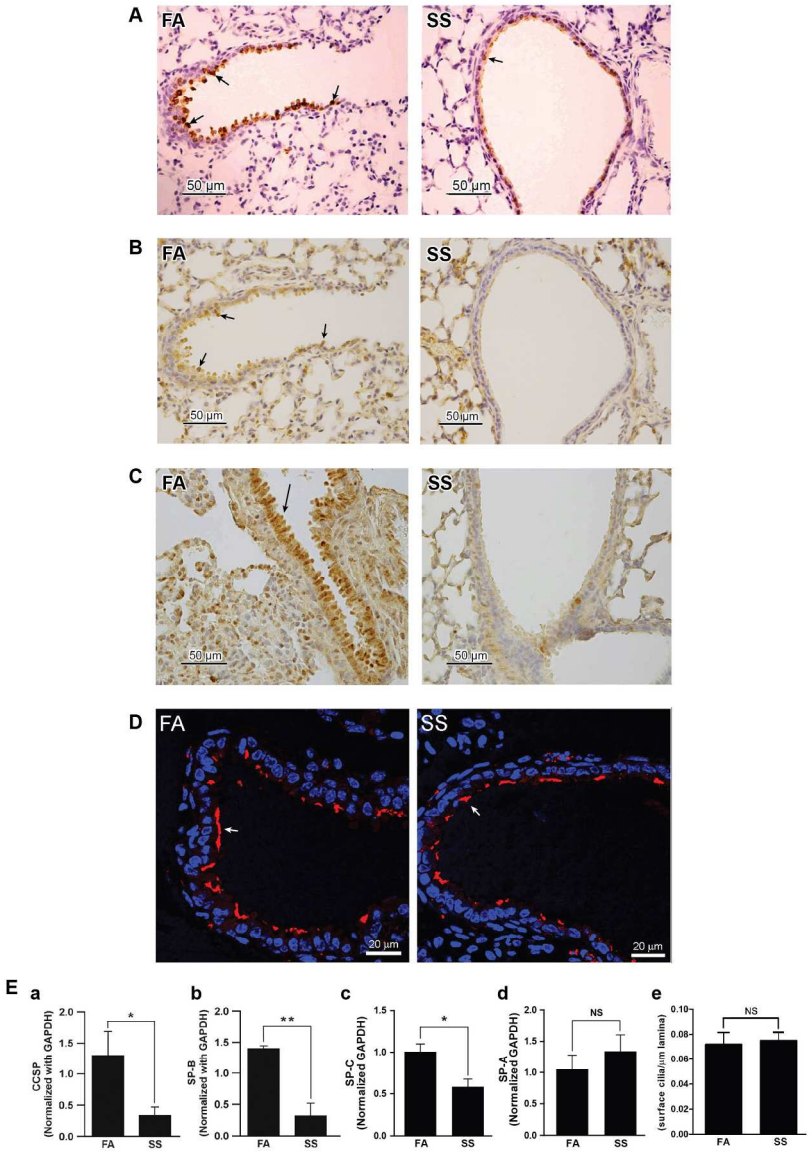

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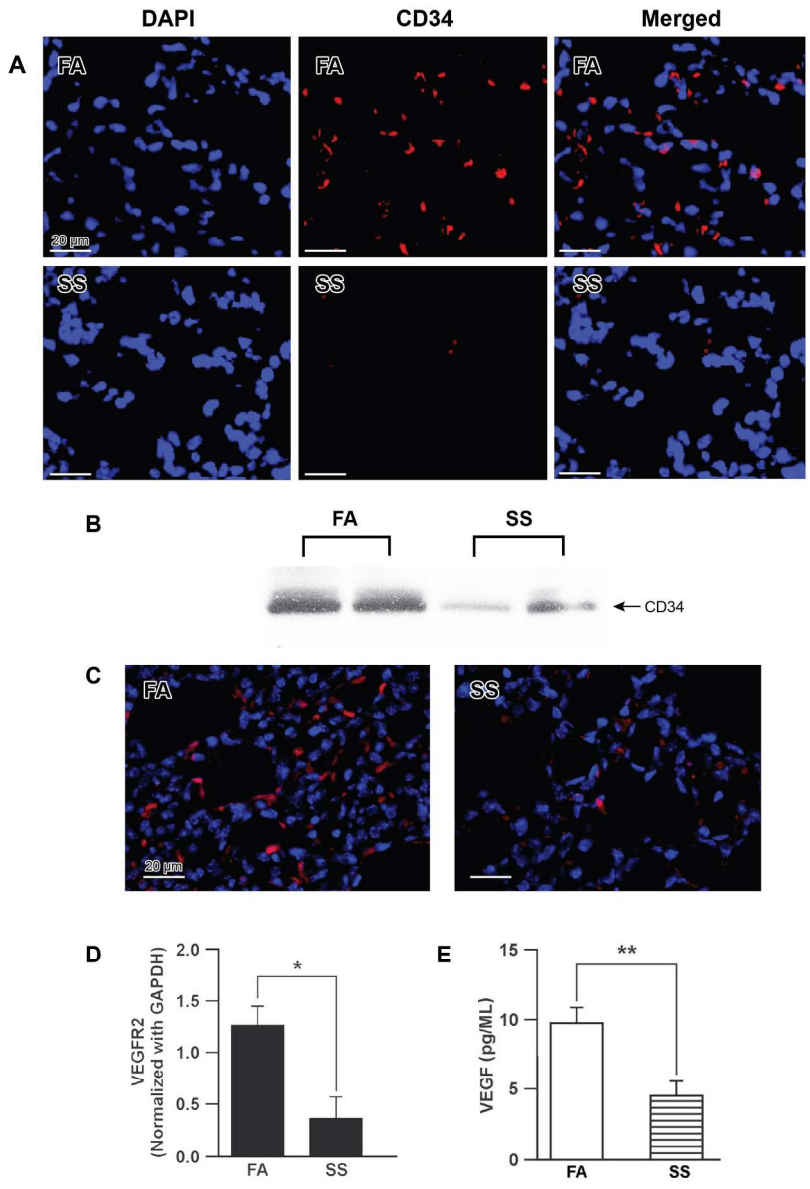

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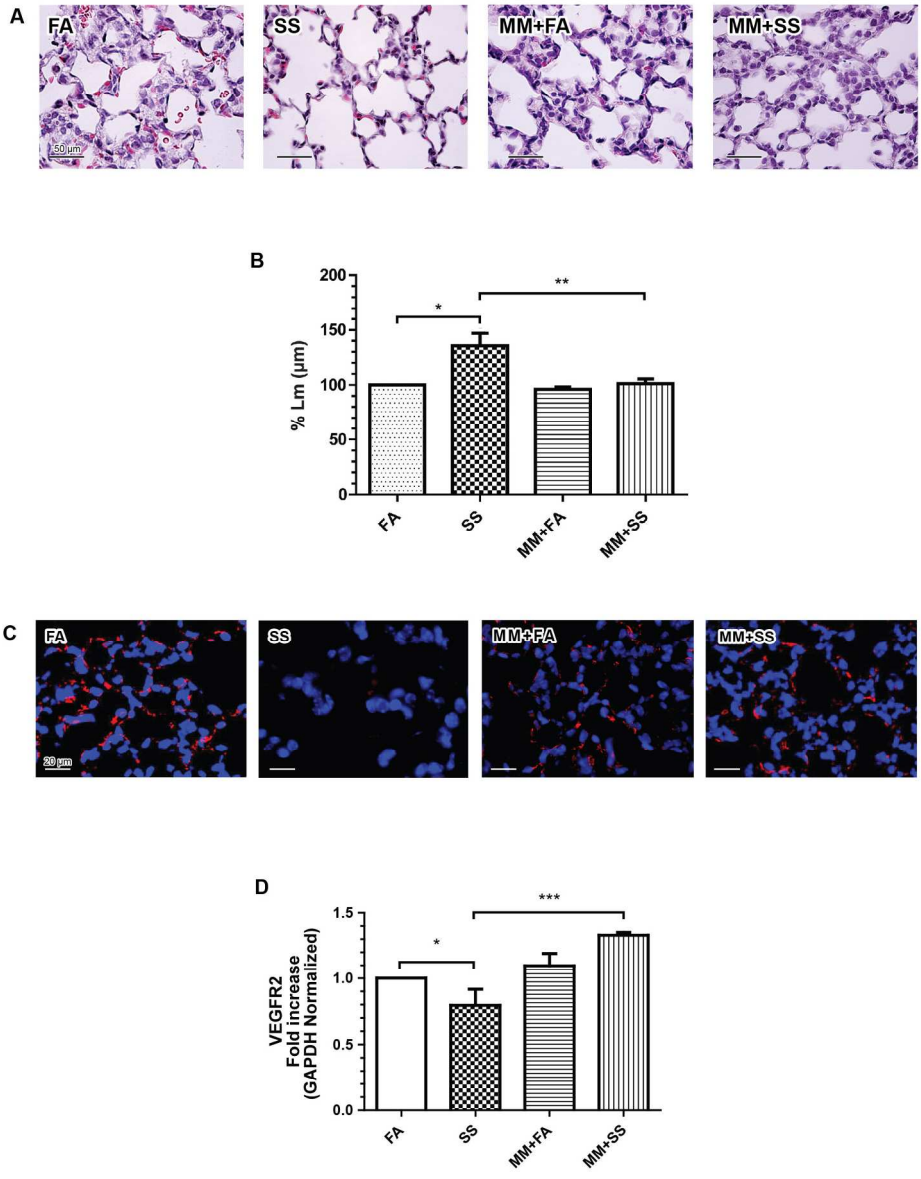

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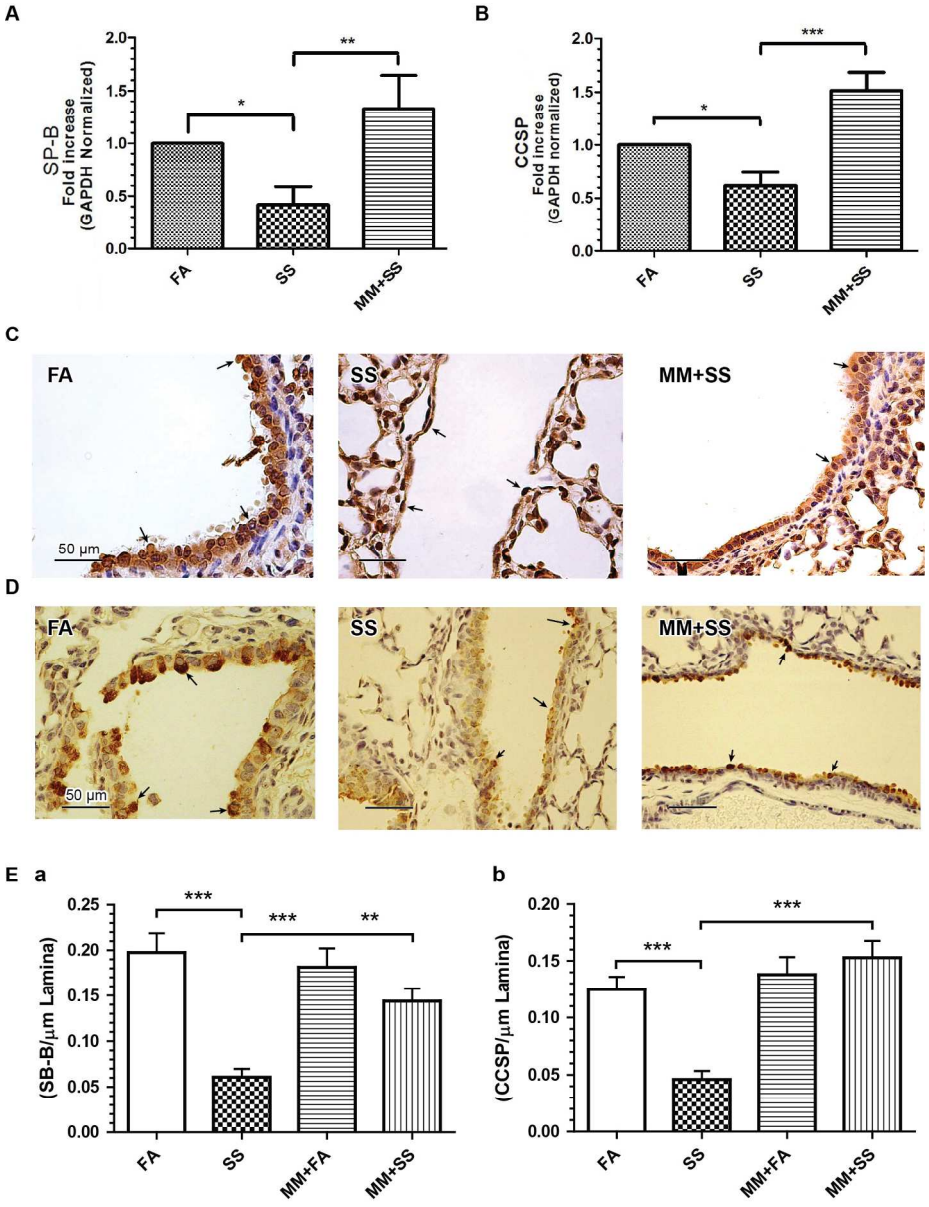

Figure 5
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